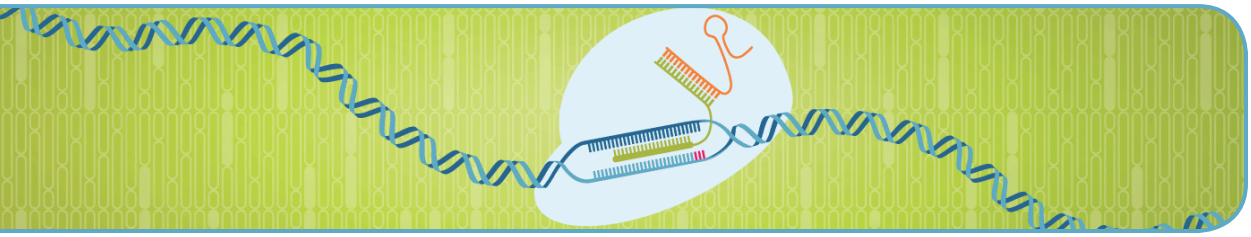
genome editing

protocol

Alt-R® CRISPR-Cas9 system:

In vitro cleavage of target DNA with ribonucleoprotein complex



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Introduction

This protocol describes how to use a Cas9 ribonucleoprotein (RNP) complex to enable *in vitro* cleavage of double-stranded, target DNA. The Cas9 RNP complex contains both an Alt-R CRISPR-Cas9 crRNA:tracrRNA guide complex and an *S. pyogenes* Cas9 endonuclease. This protocol demonstrates a method to experimentally validate the activity of CRISPR guide RNA before applying the technique in practical application.

Required materials

Reagents	Ordering information
Alt-R CRISPR-Cas9 crRNA	IDT predesigned and custom crRNA (**www.idtdna.com/CRISPR-Cas9**)
Alt-R CRISPR-Cas9 tracrRNA Alternative: Alt-R CRISPR-Cas9 tracrRNA – ATTO™ 550	IDT (cat # 1072532, 1072533, 1072534) IDT (cat # 1075927, 1075928)
Alt-R S.p. Cas9 Nuclease 3NLS Alternative: Alt-R S.p. HiFi Cas9 Nuclease 3NLS	IDT (cat # 1074181, 1074182) IDT (cat # 1078727, 1078728)
Nuclease-Free Duplex Buffer	IDT (cat # 11-05-01-12, various sizes available)
Nuclease-Free IDTE, pH 7.5 (1X TE solution)	IDT (cat # 11-01-02-02)
Nuclease-Free Water	IDT (cat # 11-04-02-01)
DNA substrate containing the target sequence	gBlocks® Gene Fragments (**www.idtdna.com/gBlocks**), or similar
For 10X Cas9 Nuclease Reaction Buffer combine: • 200 mM HEPES • 1 M NaCl • 50 mM MgCl(2) • 1 mM EDTA, pH 6.5 at 25°C	General laboratory supplier
PBS Alternative: For Cas9 Dilution Buffer combine: • 30 mM HEPES • 150 mM KCl, pH 7.5	General laboratory supplier
Proteinase K (Molecular biology grade)	General laboratory supplier

Improved enzymes

The Alt-R Cas9 enzymes have recently been further optimized to deliver even higher performance. The latest versions (V3) can be directly substituted into this protocol in place of prior Alt-R Cas9 enzymes.

Protocol

A. Prepare the double-stranded DNA template as cleavage substrate

Design your template considering the following:

- Multiple types of double-stranded DNA can be used as substrates for Cas9 cleavage. Three common examples:
  - linearized plasmid
  - purified PCR products
  - duplexed synthetic oligos
- Your template must contain a 20 nt guide sequence, followed by the Cas9 PAM site (NGG).
- The guide sequence should match the target-specific crRNA that will be used in the digestion (**step D**).
- The amount of template needed for the digestion may vary depending on the detection method (**step E**), and the template size, as shown in this table:

DNA template	Length	Final concentration	Visualization method
DNA oligo duplex	30–100 bp	2–5 uM	PAGE
gBlocks® fragment or PCR product	100–500 bp	5–50 nM	Fragment Analyzer, agarose gel
gBlocks® fragment or PCR product	500–2000 bp	2–5 nM	Fragment Analyzer, agarose gel
linearized plasmid	>2000 bp	1–2 nM	Fragment Analyzer, agarose gel

1. Ensure you are using a 10:1 molar ratio of Cas9 RNP:DNA substrate to obtain the best cleavage efficiency.
2. Resuspend or dilute the DNA substrate in Nuclease-Free Water to the required concentration.

Note: You can use the IDT resuspension calculator at **www.idtdna.com/SciTools**.

B. Create the crRNA:tracrRNA duplex

1. Resuspend each RNA oligo (Alt-R CRISPR-Cas9 crRNA and tracrRNA) in IDTE buffer to a final concentration of 100 µM.
2. Mix the two RNA oligos in equimolar concentrations in a sterile microcentrifuge tube to a final duplex concentration of 10 µM. The following table shows an example of a 10 µL final volume duplex:

Component	Amount (µL)
100 µM Alt-R CRISPR-Cas9 crRNA	1
100 µM Alt-R CRISPR-Cas9 tracrRNA	1
Nuclease-Free Duplex Buffer	8
Total volume	**10**

3. Heat the duplex at 95°C for 5 min.
4. Remove from heat and allow to cool to room temperature (15–25°C).

C. Create the RNP complex

1. Combine the crRNA:tracrRNA duplex and Cas9 enzyme in equimolar amounts.

Component	Amount (µL)
10 µM Alt-R crRNA:tracrRNA complex (from **step B4**)	10
Alt-R S.p. Cas9 Nuclease 3 NLS (61 µM stock)*	1.6
PBS†	88.4
Total volume	**100**

* All Alt-R S.p. Cas9 nucleases are provided at a stock concentration of 61 µM.

† Cas9 RNP complexes can be made in PBS, or in Cas9 dilution buffer (30 mM HEPES, 150 mM KCl, pH 7.5).

2. Incubate 5–10 min at room temperature for optimal formation of the RNP complex.

D. Perform the *in vitro* digestion reaction

1. Assemble the reaction at room temperature (15–25°C).

Component	Amount (µL)
10X Cas9 Nuclease Reaction Buffer	1
1 µM Cas9 RNP	1
100 nM DNA substrate	1
Nuclease-Free Water	7
Total volume	**10**

2. Incubate the reaction at 37°C for 60 min.
3. Add 1 µL Proteinase K (20 mg/mL) to the reaction, then incubate the mixture at 56°C for 10 min to release the DNA substrate from the Cas9 endonuclease.

E. Visualize cleaved products

Analyze the digestion by using one of the following methods:

- Agarose gel electrophoresis
- Fragment Analyzer™ System (Advanced Analytical), or similar

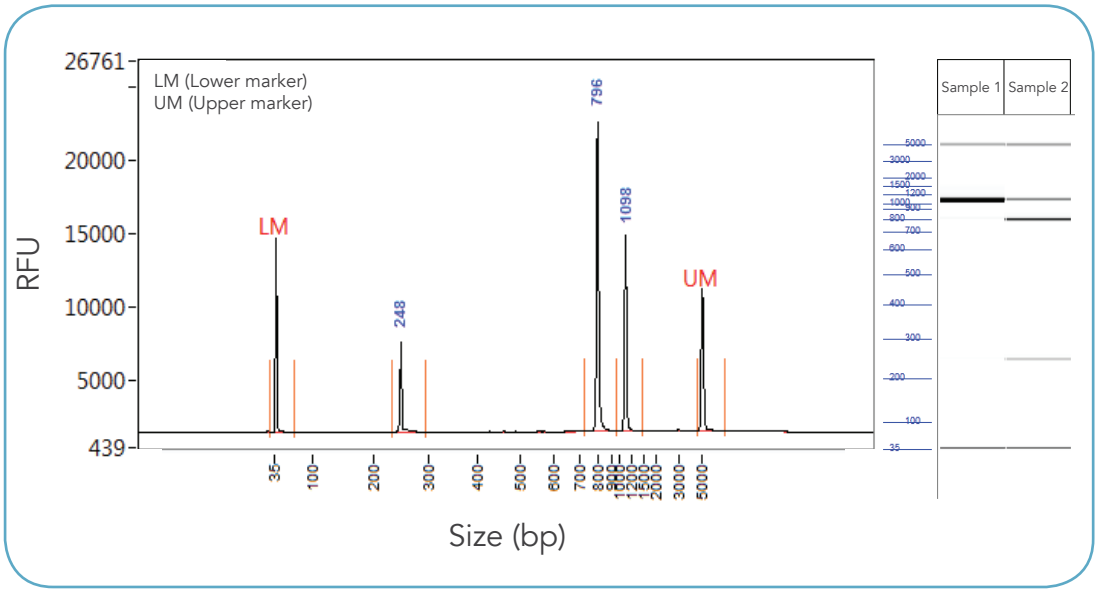

Figure 1. Sample data showing *in vitro* digestion reaction using Alt-R crRNA with CRISPR-Cas9 HPRT Positive Controls. Column-purified PCR product consisting of the Hs HPRT positive control sequence was used as a template in a 10 µL *in vitro* Cas9 digestion reaction. Sample 1 contains template without RNP. Sample 2 contains template and RNP. Digestion reactions were analyzed on a Fragment Analyzer system and a gel imaging system. Trace (left) shows results from Sample 2. Gel image (right) shows results for Samples 1 and 2.

Revision history

Version	Date released	Description of changes
1.1	May 2018	Added note about use of updated Cas9 enzymes (V3).
1	November 2017	Original protocol

Technical support:

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